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Carcinogenic N-Hydroxylaminopurine Derivatives Do Not Act as Base Analog Mutagens in 'Salmonella typhimurium'

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16. ABSTRACT

N-hydroxylaminopurines are highly mutagenic for growing as well as resting Salmonella typhimurium strain TA100 and to a lesser extent for strain TA98. Aminopurines, under similar conditions, are not mutagenic. N-methylhydroxylaminopurine, under similar conditions, exhibits only minimal activity. The results are taken to indicate that unlike non-hydroxylated aminopurines, N-hydroxylaminopurines exert their mutagenicity not by acting as base analogs but by direct covalent binding with DNA-guanine.

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Carcinogenic *N*-hydroxylaminopurine derivatives do not act as base analog mutagens in *Salmonella typhimurium*

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Summary

(1) *N*-Hydroxylaminopurines are highly mutagenic for growing as well as resting *Salmonella typhimurium* strain TA100 and to a lesser extent for strain TA98.

(2) Aminopurines, under similar conditions, are not mutagenic.

(3) *N*-Methylhydroxylaminopurine, under similar conditions, exhibits only minimal activity.

(4) The results are taken to indicate that unlike non-hydroxylated aminopurines, *N*-hydroxylaminopurines exert their mutagenicity not by acting as base analogs but by direct covalent binding with DNA-guanine.

Hydroxylaminopurines were originally synthesized and evaluated as anticancer agents (Giner-Sorolla and Bendich, 1958; Giner-Sorolla et al., 1966a, 1972). Some of them exerted a marked inhibitory activity in L1210 mouse leukemia, although they showed toxic effects in clinical trials.

N-Hydroxylaminopurines are mutagenic for a number of prokaryotic and eukaryotic species (Barrett, 1981; Brockman et al., 1979; Freese, 1968; Janion, 1978; Janion and Myszkowska, 1981; Sorenson et al., 1981); moreover, members of the group are carcinogenic to rodents (Sugiura et al., 1970; Teller, 1978, unpublished results) and induce neoplastic transformations in vitro (Barrett, 1981). In contrast, non-hydroxylated aminopurines are less mutagenic (Alderson, 1964; Barrett, 1981; Benzer, 1961; Clive et al., 1979; Freese, 1959; Ronen, 1979; Sorenson et al., 1981), do not induce neoplastic transformations in vitro (Barrett, 1981), and are not carcinogenic to rodents (Sugiura et al., 1970; Trainin et al., 1964). Heretofore, it had been assumed that purine analogs derived their mutagenic, genotoxic and carcinogenic properties from their ability to act as adenine or guanine analogs and thereby become incorporated into the cellular DNA. The apparent dichotomy in the activities of the *N*-hydroxylated and non-hydroxylated aminopurines caused us to investigate the possibility that the potent mutagenicity of *N*-hydroxylaminopurines derived from their ability to react directly with the cellular DNA in a manner analogous to that of the *N*-arylhydroxylamines, whose mutagenicity is due primarily to arylation of the C8 of the guanine

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TABLE I
MUTAGENICITY OF AMINOPURINES, *N*-HYDROXYLATED AMINOPURINES AND THEIR RIBOSIDES IN *Salmonella typhimurium*

Purine	μg/plate	Revertans/plate				
		TA98	TA100	TA100Tn5-1,8-DNP 1011	TA100Tn5-1,8-DNP 1012	TA102
Adenine	0	10	128			
	3.3	10	118			
	10	16	119			
	33	8	127			
	100	12	145			
	333	17	132			
2-Aminopurine	0	10	128			
	3.3	12	115			
	10	9	132			
	33	8	113			
	100	9	143			
	333	17	161			
2,6-Diaminopurine	0	13	66			
	3.3	13	52			
	10	10	51			
	33	13	59			
	100	12	69			
	333	13	68			
Adenosine	0	13	66			
	3.3	9	62			
	10	8	54			
	33	9	56			
	100	11	68			
	333	11	60			
6-*N*-Hydroxylaminopurine	0		86	163	131	
	0.01		98	183	145	
	0.03		142	304	276	
	0.1		396	705	579	
	0.3		335	298	192	
	1		127	84	93	
6-*N*-Hydroxylaminopurine riboside	0	16	86	163	131	162
	0.03		121	169	155	
	0.1	23	194	304	346	157
	0.3	73	322	492	502	156
	1.0	100	95	101	121	158
	3.3	10	77	76	124	179
	10					249
2-Amino-6-*N*-hydroxyl aminopurine riboside	0		86	163	131	
	0.01		360	477	520	
	0.03		612	904	953	
	0.1		1257	1480	1647	
	0.3		1468	1945	1697	
	1.0		1583	1870	1786	

TABLE I *(continued)*

Purine	μg/plate	Revertans/plate				
		TA98	TA100	TA100Tn5-1,8-DNP 1011	TA100Tn5-1,8-DNP 1012	TA102
6-*N*-Methylhydroxylamino-purine riboside	0		86	163	131	
	3		63	87	134	
	10		95	131	128	
	33		109	152	194	
	100		136	197	287	
	333		167	269	328	
Sodium azide	10		776	730	804	
2-Nitrofluorene	33	1226				
β-Propiolactone	0.1 μl					618

Results of representative mutagenicity assay. Each concentration was tested in triplicate.

moiety in DNA (Beland et al., 1983; Howard et al., 1983; Rosenkranz and Mermelstein, 1983). The present findings suggest that this is indeed the case.

Materials and methods

Chemicals

Adenine (6-aminopurine), adenosine (adenine riboside, 9-β-D-ribofuranosyl adenine), 2-aminopurine and 2,6-diaminopurine were obtained from Sigma Chemical Co. The 6-*N*-hydroxylaminopurines were synthesized by previously described procedures: 6-*N*-hydroxylaminopurine (Giner-Sorolla and Bendich, 1958), 9-β-D-ribofuranosyl-6-*N*-hydroxylaminopurine (6-*N*-hydroxylaminopurine riboside) (Giner-Sorolla et al., 1966a), 9-β-D-ribofuranosyl-2-amino-6-*N*-hydroxylaminopurine (2-amino-6-*N*-hydroxylaminopurine riboside) (Giner-Sorolla et al., 1966b) and 9-β-D-ribofuranosyl-6-*N*-methylhydroxylaminopurine (6-*N*-methylhydroxylaminopurine riboside) (Ginger-Sorolla et al., 1968).

Bacterial strains

Salmonella typhimurium strains TA1530, TA1535, TA100, TA102 and TA98 were obtained from Dr. Bruce N. Ames, University of California, Berkeley (Ames et al., 1975; Maron and Ames, 1983; Levin et al., 1982). Strains TA100Tn5-1,8DNP 1011 and TA100Tn5-1,8DNP 1012 are derivatives of TA100 which are deficient in an esterification enzyme required for the expression of the mutagenicity of a number of *N*-arylhydroxylamines (McCoy et al., 1983).

Standard mutagenicity assay

The Salmonella mutagenicity assay was carried out as described previously (Ames et al., 1975; Maron and Ames, 1983), with the modification that the histidine content of the agar overlay was reduced to 0.01 μM (Dunkel et al., 1984). Every concentration was tested in triplicate on at least two separate occasions. Control mutagens for TA1530, TA1535, TA100, TA100Tn5-1,8DNP 1011 and 1012 included sodium azide and ethyl methanesulfonate, for TA98 2-nitrofluorene and for TA102 β-propiolactone. 1,8-Dinitropyrene was used to verify the non-responsiveness of esterification-deficient strains TA100Tn5-1,8DNP 1011 and TA100Tn5-1,8DNP 1012. Mutagenic activity was calculated from the linear, ascending portion of the dose-response curve.

Mutagenicity to non-growing Salmonella

Overnight cultures of *Salmonella typhimurium* growing in trypticase soy broth (BBL) were harvested by centrifugation (12 000 × *g*; 10 min), washed with minimal medium and resuspended in

the same medium at 1/100 the original volume. 0.16-ml portions of the bacterial cultures received 0.05-ml amounts of various dilutions of the test chemicals and 0.79 ml of minimal medium. The cultures were incubated with aeration for 1 h at 37°C, wherupon they were chilled in an ice bath (4°C), the cells harvested by centrifugation, washed with cold minimal medium and resuspended in 1.0 ml of the same medium. Triplicate 0.3-ml amounts were added to 2 ml soft agar and processed for the determination of revertants to histidine independence. Neither the liquid nor the agar-containing minimal media contained any histidine. Other portions of the remaining cultures were serially diluted and 0.1-ml quantities plated onto the surface of trypticase soy agar plates, incubated at 37°C for 24 h, where upon surviving colonies were enumerated.

Results and discussion

The results of representative Salmonella mutagenicity asays are detailed in Table 1 and summarized in Table 2. They indicate that, as expected, adenine and its 9-β-D-ribofuranosyl derivative adenosine are devoid of activity. It was also found that in the standard Salmonella mutagenicity assay neither of the two base analogs, 2-aminopurine or 2,6-diaminopurine, was mutagenic, although their mutagenicity for bacteriophage and for other strains of *Salmonella typhimurium* has been reported (Ronen, 1979; MacPhee et al., 1983). On the other hand, 6-*N*-hydroxylaminopurine and its riboside (i.e., 9-β-D-ribofuranosyl-6-*N*-hydroxyl-aminopurine) as well as the riboside of 2-amino-6-*N*-hydroxylaminopurine, were highly mutagenic for strain TA100, the strain used to detect base substitution mutations (these *N*-hydroxylaminopurines were also mutagenic for strains TA1530 and TA1535, which also detect base substitution mutations; results not shown). 2-Amino-6-*N*-hydroxylaminopurine riboside was the most mutagenic species tested. This nucleoside analog was also tested in strain TA98, the strain used to detect frameshift mutagens, in which strain it was moderately active. *N*-Methylation of *N*-hydroxylaminopurine (*i.e.*, 6-*N*-methylhydroxylaminopurine riboside) resulted in virtual elimination of the mutagenicity.

The lack of mutagenicity of the non-*N*-hydroxylated aminopurines suggests that the mutagenicity of the *N*-hydroxylaminopurines is indeed due to covalent binding with DNA. Mutagenicity induced by arylhydroxylamines is

TABLE 2
MUTAGENICITY OF *N*-HYDROXYLAMINOPURINES FOR *Salmonella typhimurium*: STANDARD ASSAY

Purine	Revertants/nmole				
	TA100	TA100Tn5-1,8-DNP 1011	TA100Tn5-1,8-DNP 1012	TA98	TA102
Adenine	0			0	
2-Aminopurine	0.02			0	
2,6-Diaminopurine	0			0	
Adenosine	0			0	
6-*N*-Hydroxylaminopurine	287	836	691		
6-*N*-Hydroxylaminopurine riboside	216	303	390		
2-Amino-6-*N*-hydroxylamino-purine riboside	3242	3602	4120	25	2.7
6-*N*-Methylhydroxylamino-purine riboside	0.08	0.09	0.08		
N-2-Hydroxylaminofluorene[a,b]	27	8	5	1185	9
4-Nitroquinoline-1-oxide[b]	824		1078	155	75
N-2-Hydroxylaminonaphthalene[a]	15			3.7	2.2

[a]Data taken from Massaro et al., 1983.
[b]Data taken from McCoy et al., 1983.

TABLE 3
MUTAGENICITY OF N-HYDROXYLAMINOPURINES: EXPOSURE OF NON-GROWING CELLS

Purine	μg/ml	Revertants/ plate	Revertants/ml	Survivors $\times 10^{-7}$/ml	Revertants/ 10^7 survivors
Strain TA100					
6-*N*-Hydroxylamino-purine	0	96	320	143	2.23
	0.1	105	350	85	4.12
	0.3	157	523	109	4.80
	1.0	255	850	91	9.34
	3.3	370	1233	49	25.17
	10	400	1333	77	17.31
6-*N*-Hydroxylamino-purine riboside	0	96	320	143	2.23
	0.1	97	323	99	3.27
	0.3	102	340	127	2.68
	1.0	119	397	142	2.79
	3.3	159	530	137	3.87
	10	179	597	115	5.19
2-Amino-6-hydroxyl-aminopurine riboside	0	174	580	181	3.20
	0.1	475	1583	183	8.65
	0.3	770	2566	176	14.58
	1.0	1219	4063	210	19.35
	3.3	1411	4703	156	30.15
	10	1349	4496	163	27.58
Strain TA98					
6-*N*-Hydroxylamino-purine	0	10	33.3	99	0.34
	0.1	12	40.0	101	0.40
	0.3	10	33.3	103	0.32
	1.0	18	60.0	91	0.66
	3.3	21	70.0	124	0.57
	10	19	63.3	89	0.71
6-*N*-Hydroxylamino-purine riboside	0	10	33.3	99	0.34
	0.1	11	36.7	114	0.32
	0.3	10	33.3	105	0.32
	1.0	10	33.3	127	0.26
	3.3	12	46.0	107	0.37
	10	16	533	82	0.65

thought to involve primarily the C8 of guanine (Beland et al., 1983; Howard et al., 1983). The data are consistent with a similar mechanism being involved in the mutagenicity of *N*-hydroxylaminopurines; this is supported by the mutagenic specificity of these purines for strains TA100, TA1530 and TA1535, which have a GC base pair at their mutational site. On the other hand, the mutagenicity was minimal in strain TA102, which has an AT base pair as the mutational target (Levin et al., 1982; Maron and Ames, 1983; Barner et al., 1982; Isono and Yourno, 1974).

It is thought that arylation results from the non-enzymic conversion of *N*-hydroxylaminoarenes to the corresponding arylnitrenium ions which are the ultimate nucleophiles (McCoy et al., 1983). Consistent with such a hypothesis for the basis of the activity of *N*-hydroxylaminopurine is the finding that the substituted analog, unable to undergo conversion to a nitrenium ion (i.e., 6-*N*-methylhydroxylaminopurine riboside) is practically devoid of mutagenicity.

Some arylhydroxylamines require esterification prior to conversion to the corresponding arylnitrenium ions. These chemicals exhibit greatly reduced mutagenicity in Salmonella strains devoid of the needed esterification enzyme. The mutagenicity of other arylhydroxylamines is increased when tested in such esterification-deficient strains (Rosenkranz et al., 1985). This is taken to mean that esterification decreases the rate of nitrenium ion formation. Chemicals whose activation to mutagenic species does not involve esterification (i.e., non-arylhydroxylamines such as metabolites of PAHs and direct-alkylating agents) are equally active in esterification-proficient and -deficient strains. None of the *N*-hydroxylaminopurines tested showed decreases when tested in the esterification-deficient strains TA100Tn5-1,8DNP 1011 and TA100Tn5-1,8DNP 1012. On the contrary, the absence of esterification activity enhanced mutagenicity, indicating that *N*-hydroxylaminopurines formed the corresponding nitrenium ions without a requirement for esterification.

The fact that *N*-hydroxylaminopurines also showed some activity in strain TA98 (Tables 1–3) supports the covalent binding hypothesis; base substitution mutations induced by base analogs would not be detected in TA98. It has, however, been found that mono- and bicyclic *N*-hydroxylamines, which react with DNA to form adducts, while being more mutagenic for TA100, are also active in TA98 (Rosenkranz and Mermelstein, 1983; Table 2). On the other hand, arylhydroxylamines with three or more rings are primarily mutagenic in TA98. In further support of a direct reaction of the purinylnitrenium ion with the C8 of DNA-guanine is the fact that the mutational site in TA98 consists of 8 alternating GC base pairs (Isono and Yourno, 1974; Maron and Ames, 1983).

The mutagenic potencies of the *N*-hydroxylaminopurines in TA100 are of the same order as that of 4-nitroquinoline-1-oxide, which is reduced to a *N*-hydroxylamino intermediate (McCoy et al., 1981); they are much higher than that of *N*-2-naphthylhydroxylamine, the metabolite of the human carcinogen 2-naphthylamine (Table 2).

If indeed the mutagenicity of the *N*-hydroxylaminopurines is not due to base incorporation but to direct adduct formation, then it can be predicted that these chemicals will also be mutagenic for non-growing cells, which do not synthesize DNA and which therefore cannot incorporate base analogs. Indeed, pulsed exposure of non-growing Salmonella to *N*-hydroxyaminopurine resulted in mutagenicity, thus further confirming the proposed mechanism of action of *N*-hydroxylaminopurines. Conversely, the non-hydroxylated aminopurines were without activity (data not shown).

These results, which indicate that the carcinogenic *N*-hydroxylaminopurines have a different mechanism of action than the aminopurines, i.e., direct adduct formation versus base incorporation, suggest that the ability of candidate anti-neoplastic purine analogs to form DNA adducts be determined as it may be an indication of carcinogenic potential as well.

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